REMARKS

Applicants sincerely appreciate the Examiner's time on the phone during a telephone interview on February 19, 2010 during which claim amendments were discussed to more clearly distinguish the particular novel features of the invention from general pyrosequencing methods. Specifically, amendments adding the specific steps for designing the dispensation order so that it matches one sequence at a time and a subsequent step of dispensing the nucleotides in the predetermined order in a multiplex reaction so that two sequential pyrograms representing two separate sequences are produced instead of one program with combined peaks from the sequenced nucleic acids which is the way prior art shows use of multiplex pyrosequencing.

Accordingly, Applicants have amended claims 1, 18, 22, and 24 to add steps (b) and (c) as set forth above.

Applicants respectfully submit that the amendments are supported by the specification or are clerical or grammatical, and therefore, no new matter has been introduced by the amendments and their entry is respectfully requested.

Applicants now turn to the specific rejections.

The Examiner rejected claims 1-31, 35-36, 38 and 39 under 35 U.S.C. §103(a) as allegedly obvious over Nadeau et al. (U.S. Patent No. 5,840,487) in view of Therianos et al. (U.S. Patent Application Publication No. 20050089862), Haemmerle et al. (U.S. Patent No. 5,858,658), Antonarakis et al. (U.S. Patent Application Publication No. 20030054368), and Ronaghi et al. (Journal of Chromatography B, vol. 782, pp. 67-72, 2002).

Applicants respectfully disagree and submit that the rejection should be withdrawn for the following reasons.

As discussed during the telephone interview on February 19, 2010 and again on telephone on March 22, 2010, none of the cited prior art references teach or suggest a quantification method that uses multiplex pyrosequencing and allows one to simply compare the peak heights to obtain the result (see exhibit A submitted with the Amendment dated September 21, 2009).

The specific advantage of the present method is to provide separate and independent pyrograms (**sequential pyrograms**) for each nucleic acid in a multiplex reaction. Such independent pyrograms then allow one to directly compare the peak heights from different

reaction products and therefore provide quantitative information of target nucleic acid compared to a control the amount of which is known in the original sample.

Nothing in the prior art references teaches or suggests how such a comparison could be performed in a multiplex pyrosequencing reaction.

While multiplex pyrosequencing reactions have been described in prior art, all of them rely on obtaining on "peak set" per reaction. In all the cited prior art references the peaks in the program are "uneven" because the one program represents a mixture of sequences in the multiplex reaction. Such uneven peaks are impossible to evaluate for quantification purposes.

The present claims provide a novel concept of sequentially resolving the multiplex reactions. This results in "even peaks", because each program represents only one nucleic acid present in the multiplex reaction.

This significant difference is shown graphically in Exhibit A submitted previously.

The multiplex pyrosequencing described in Ronaghi suffers from this specific "uneven peaks" -defect. Ronaghi specifically describes that "[p]yrograms obtained from multiplex pyrosequencing reflect **number of nucleotides** incorporated by all primers and can easily be **deconvoluted to singleplex** pyrosequencing data" (p. 69, emphasis added). This passage shows that Ronaghi does not envision a multiplex reaction without the specific "deconvolution step" to resolve each sequence from one pyrogram. While such "deconvolution" step may be easy for a method of simple sequencing, such deconvoluted peaks cannot be used for the purposes of nucleic acid quantification.

The method of the present claims does not need the deconvolution step because by predetermining the order the nucleiotides are dispensed, one produces sequential "singleplex pyrograms" directly from the multiplex reaction mixture, each such program representing the sequence from one nucleic acid sequence of the multiplex sequences present in the reaction. Therefore, the peak areas are also true to the original amount of the sequence in the starting mixture and can be quantitatively compared to each other.

Antonarakis suffers from the same problem and Ronaghi. As explained in the September 21, 2009 amendment, also Antonarakis produces **one single pyrogram,** which represents the **combination of the sequences** present in the analyzed mixture. Thus, each peak represents a combination of the two sequences except for those locations where the sequences of the two differ.

In contrast, the presently claimed method produces **sequential reaction products, such as pyrograms** from the two different primers **representing each of the samples separately**. Each peak thus represents the accurate amount from one single sequence.

Accordingly, neither Antonarakis nor Ronaghi teach how one could **produce separate pyrograms representing each sequence alone from a multiplex reaction**, which is, as explained already above, the key invention of the present claims.

Applicants further refer the Examiner to a book describing the state of the art of multiplex pyrosequencing: "Multiplex Pyrosequencing® for DNA Variation Analysis, Book Series Methods in Molecular Biology™ , ISSN 1064-3745 (Print) 1940-6029 (Online), Volume Volume 373, Book Pyrosequencing Protocols, Publisher Humana Press" published in 2007. Applicants attach a description from the book regarding multiplex assay design for the Examiner to review as Exhibit A. The excerpt makes it clear that even in 2007, one skilled in the art did not contemplate producing sequential pyrograms (see, e.g. Figure 1 in Exhibit A).

Moreover, while the text describes that the design of the dispensation order is important, it does not provide a teaching how to design a dispensation order that would result in absence of the composite peaks (peaks representing nucleotides from two different templates). Specifically, the text states that "peaks from each polymorphic and at least one nonpolymorphic sites of the same primer should not overlap with the peaks from the other extension reactions" but the chapter goes on stating that "other peaks in the multiplex incorporation pattern may be composite peaks". The only guidance the book gives about optimizing the dispensation order is that the "general scheme of finding an optimal dispensation order is essentially iterative".

Contrary to the description in Exhibit A, the present claims set forth two specific conditions not described in other multiplexing protocols, namely, (1) that the sequence of the control must differ from the sequence of the target, and (2) that the dispensation order is determined by the order of the nucleotides in these sequences so that only one sequence at a time gets extended and thus results in sequential pyrograms, not one composite pyrogram.

In view of the above, Applicants respectfully submit that the claims are in condition for allowance. Early and favorable consideration is sincerely solicited. Applicants appreciate the Examiner's offer to contact them to expedite prosecution in case additional questions arise during further examination.

If any fee deficiencies are associated with this submission, the Commissioner is authorized to debit such deficiencies to the Nixon Peabody Deposit Account No. 50-0850. Any overpayments should be credited to the same Deposit Account.

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Respectfully submitted,

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